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Sunshine virus in Australian pythons

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Abstract

Sunshine virus is a recently discovered novel paramyxovirus that is associated with illness in snakes. It does not phylogenetically cluster within either of the two currently-accepted paramyxoviral subfamilies. It is therefore only distantly related to the only other known genus of reptilian paramyxoviruses, *Ferlavirus*, which clusters within the *Paramyxovirinae* subfamily. Clinical and diagnostic aspects associated with Sunshine virus are as yet undescribed. The objective of this paper was to report the clinical presentation, virus isolation, PCR testing and pathology associated with Sunshine virus infection. Clinical records and samples from naturally occurring cases were obtained from two captive snake collections and the archives of a veterinary diagnostic laboratory. The clinical signs that are associated with Sunshine virus infection are localised to the neurorespiratory systems or are non-specific (e.g. lethargy, inappetence). Out of 15 snakes that were infected with Sunshine virus (detected in any organ by either virus isolation or PCR), the

virus was isolated from four out of ten (4/10) sampled brains, 3/10 sampled lungs and 2/7 pooled samples of kidney and liver. In these same 15 snakes, PCR was able to successfully detect Sunshine virus in fresh-frozen brain (11/11), kidney (7/8), lung (8/11) and liver (5/8); and various formalin-fixed paraffin-embedded tissues (7/8). During a natural outbreak of Sunshine virus in a collection of 32 snakes, the virus could be detected in five out of 39 combined oral-cloacal swabs that were collected from 23 of these snakes over a 105 day period. All snakes that were infected with Sunshine virus were negative for reovirus and ferlavirus by PCR. Snakes infected with Sunshine virus reliably exhibited hindbrain white matter spongiosis and gliosis with extension to the surrounding grey matter and neuronal necrosis evident in severe cases. Five out of eight infected snakes also exhibited mild bronchointerstitial pneumonia. Infection with Sunshine virus should be considered by veterinarians investigating disease outbreaks in snakes, particularly those that are associated with neurorespiratory disease.

Keywords

Reptile; snake; python; Sunshine virus; paramyxovirus; virus

Introduction

A range of pathogenic viruses have been detected in snakes throughout the world and for overviews of these viruses and their associated diseases, the interested reader is directed to the

excellent reviews by Wellehan and Johnson (2005), Jacobson (2007) and Marschang (2011). Of the viruses that have been reported in snakes, paramyxoviruses are particularly important since disease outbreaks of significant morbidity and mortality have been detected in Europe, USA and Brazil (Folsch and Leloup, 1976; Jacobson et al., 1992; Kolesnikovas et al., 2006). Prior to the discovery of Sunshine virus, all phylogenetically-characterised reptilian paramyxoviruses had clustered within the recently-accepted paramyxoviral genus, *Ferlavirus* (Marschang et al., 2009; ICTV, 2012). Australian native species (eg. *Morelia sp*.) are present in herpetological collections all over the world, and have been described as being afflicted with as yet poorly described, possibly paramyxoviral neurological disease (Boyer et al., 2000; Jacobson, 2005). In contrast to the rapidly-expanding knowledge of snake virology that exists elsewhere in the world, snake virology in Australia remains in its infancy.

For many years, Australian snakes, especially pythons from eastern Australia, have presented to veterinarians with neurological and/or respiratory disease (Rose et al., 2005). The diagnostic tests available to these practitioners to investigate infectious aetiologies have been limited and the cause of disease for many animals has remained elusive. Information is limited to only a few reports containing limited information.

Reovirus particles have been identified by electron microscopy in the brains of Australian snakes with neurological dysfunction (Rose et al., 2005).

In 1998, Carlisle-Nowak et al. reported on inclusion body disease (IBD) in two Australian pythons. Diagnosis was based on clinical signs and histopathological findings that were consistent with IBD.

Evidence for the presence of ferlaviruses in Australia is tenuous. Serology from some captive snakes has been positive for ferlaviruses but the details of testing are not provided (Rose et al., 2005). Histopathology consistent with ferlaviruses has been briefly described in snakes within Australia (Sullivan, 2005). However, there are no reports concerning the isolation, visualisation by electron microscopy, or molecular detection by PCR, of ferlavirus in an Australian snake.

In 2008, an outbreak of neurorespiratory disease occurred in a collection of 70 native Australian python species from the Sunshine Coast of Queensland, Australia (approximately 100 kilometres north of Brisbane). The entire collection was euthanased and samples from 17 of these snakes were opportunistically-retrieved by the attending veterinarian for virus isolation. A syncytium-forming virus was isolated using viper heart cells (VH2) but a range of PCR primers for the detection of ferlaviruses (genus-specific), paramyxoviruses (family- and subfamily-specific) and reoviruses failed to identify this isolate (Hyndman et al., 2012). Biochemical testing of this isolate provided largely equivocal results due to the low viral titre ($TCID_{50}$ = $10^{2.75}$ mL^{-1}). As such, any haemagglutinating and/or neuraminidase activity of this isolate could not be determined. Illumina® high-throughput sequencing revealed this new virus to be a novel

paramyxovirus (GenBank accession number: JN192445) that was named Sunshine virus after the geographical origin of this first isolate. Phylogenetic analysis supported the assignment of Sunshine virus as a member of the family *Paramyxoviridae* but as being distinct from the two existing subfamilies: *Paramyxovirinae* and *Pneumovirinae*. The divergence between the attachment protein sequences of Sunshine virus and other paramyxoviruses, did not allow the nature of the Sunshine virus attachment protein (H, HN or G) to be determined by molecular methods.

This report expands the knowledge of Australian and international snake virology by describing the clinical signs, gross pathology, histological findings and the results of PCR testing associated with Sunshine virus infection.

Materials and Methods

Sample Collection

Samples that were analysed in this investigation came from three sources: two Australian captive collections and the archives of an Australian veterinary diagnostic laboratory.

Collection 1

In 2008, all the snakes (70 native Australian pythons from the genera *Aspidites*, *Morelia* and *Antaresia*) in a private collection were humanely euthanased in response to an outbreak of neurorespiratory disease. During the outbreak, but before destocking, two snakes died and a further 14 were displaying signs

of neurorespiratory disease (further historical details of the events leading up to destocking are presented in Supplementary Figure 1). In total, freshly frozen samples from 17 livers, kidneys and lungs, 16 brains and 13 serum samples were collected from 17 snakes and submitted to Murdoch University for virus isolation and PCR testing. Snakes were selected for sample collection based on clinical signs and/or which snakes they had been in direct contact with.

Collection 2

In 2011, sporadic cases of neurological and other non-specific signs of disease occurred in a collection of 32 snakes (20 Australian pythons from the genera *Morelia* and *Antaresia*; four exotic boas; and eight Australian elapids). Cloacal and oral swabs were opportunistically sampled on multiple occasions from 23 of these snakes. In addition, from one snake that was euthanased, fresh samples of brain, liver, kidney and lung were collected. Samples were submitted to Murdoch University for PCR testing.

None of the eight venomous snakes from this collection (*Pseudechis*, the black snakes; *Oxyuranus*, the taipans; *Acanthophis*, the death adders; and *Notechis*, the tiger snakes) were showing overt signs of ill-health and for safety reasons, these snakes were not sampled.

For combined oral-cloacal swabs, a cotton-tipped applicator was pre-moistened in isotonic saline (or Hartmann's solution) and then the inside of the mouth (especially the glottis) and the cloaca were swabbed. Oral-only and cloacal-only swabs were also taken from a subset of the snakes. All swab tips were broken off into sterile

containers, submerged in isotonic saline (or Hartmann's solution) and then sent to Murdoch University for PCR testing. For all snakes that were PCR tested for Sunshine virus using swab samples, the combined oral-cloacal swab was tested first. If a snake tested positive, and individual swabs were available, the individual swabs were then tested to determine whether the oral-only and/or the cloacal-only swabs were positive.

Veterinary Diagnostic Laboratory

Archives of the Berrimah Veterinary Laboratories (BVL, Northern Territory, Australia) were searched for snake submissions that had histopathological evidence of neurological disease suggestive of a viral aetiology. In total, nine snakes from four collections met the inclusion criteria. Case records included historical and clinical data as well as details of the full necropsy that had been performed on each snake at the laboratory. Tissue sections that had been processed in standard fashion for histological examination and stained with haematoxylin and eosin were retrieved for review and a detailed description by one author (C.S.). In all snakes, organs examined histologically included representative sections of the brain (a parasagittal section starting from the anterior extent of the forebrain, variably including the olfactory bulbs, that extended posteriorly to the junction of the hindbrain with the spinal cord), lung, kidney, heart, stomach, and the small and large intestines. Additionally, sections of the liver, spleen and exocrine pancreas were also examined histologically in most snakes and a section of the cervical spinal cord was examined in two snakes. Paraffin blocks

were sent to Murdoch University where 10µm sections were cut and collected into sterile microcentrifuge tubes for PCR testing. A new microtome blade was used for the paraffin block(s) created from each snake. Paraffin blocks for each snake contained brain and lung plus a variety of other organs. Fresh frozen brain and/or lung were available for virus isolation from five of these nine cases.

Formalin-fixed paraffin-embedded (FFPE) brain sections from six snakes that had died from recent road trauma served as negative controls. These sections were screened for paramyxovirus infection (Sunshine virus and ferlavirus) by PCR and were also examined histologically to help distinguish subtle neuropathology from normal variation.

Virus Isolation

Fresh-frozen tissues were processed for virus isolation as previously reported (Hyndman et al., 2012).

Polymerase Chain Reaction

Containers that contained swab tips immersed in either isotonic saline or Hartmann's solution, were vigorously vortexed for at least 30 seconds and then a 200µL aliquot of the saline or Hartmann's solution was used for nucleic acid extraction using the Purelink™ Viral RNA/DNA Mini Kit (Cat. No. 12280-050, Invitrogen, Victoria) according to the manufacturer's instructions. Fresh-frozen tissues were processed using the MELT™ Total Nucleic Acid Isolation System (Cat. No. AM1983, Ambion, Texas) according to the manufacturer's instructions. Total nucleic acid from both extraction

procedures was eluted into 30µL of elution buffer. 13.5µL of total nucleic acid was added to 1µL of random hexamers (100ng/µL) and 1µL of dNTPs (10mM) and incubated at 65^0C for five minutes. 0.5µL of Superscript® III reverse transcriptase (200 units/µL, Cat. No. 18080-044, Invitrogen, Victoria) and 4µL of 5x buffer were then added to make a final volume of 20µL which was then incubated at 25^0C for five minutes, 45^0C for 45 minutes and 70^0C for 15 minutes. For PCR amplification, 1µM (final concentration) of SunshineS2 (5'-TTCAAGGAGATAACCAGG) and SunshineAS1 (5'-ATTCAACATCTGGGGTC) (amplifies a 357 nucleotide segment of the viral polymerase gene), was added to 1µL of cDNA and then Platinum® PCR Supermix (Cat. No. 11306-016, Invitrogen, Victoria) was added to bring the final reaction volume to 20µL. Cycling conditions were as follows: 94^0C x 2min, 40 x (94^0C x 20s, 45^0C x 45s, 72^0C x 30s). PCR products were visualised using agarose gel electrophoresis and sequencing of appropriately-sized PCR products was accomplished using an AB3730xl DNA Analyser (Applied Biosystems, California) after PCR products were excised from agarose gels and purified using a Purelink™ Quick Gel Extraction Kit (Cat. No. K210012, Invitrogen, Victoria).

For formalin-fixed paraffin-embedded (FFPE) samples, total nucleic acid was recovered as per the methods described for fresh-frozen tissues, but with minor modification. Samples were first deparaffinised in two washes of xylene and then the xylene was cleared with two washes of ethanol. Next, deparaffinised tissues were digested overnight at 50^0C in the digestion cocktail provided

with the Ambion kit. RNA was reverse transcribed into cDNA as per the methods described for fresh-frozen tissues. For PCR amplification, the primer pair, SunshineS1 (5'GGAAAGGGAGGTCTATG) and SunshineAS1 (5'ATTCAACATCTGGGGTC), was used for the detection of Sunshine virus because of the smaller amplicon that is produced (153 nucleotides).

An isolate of Sunshine virus (GenBank accession number: JN192445) served as a positive control for all Sunshine virus PCRs.

At least one Sunshine virus PCR-positive sample from each Sunshine virus PCR-positive snake was tested for ferlavirus and reovirus. For ferlavirus PCR, the primer pair, ferlavirusqS2 (5'GTTATGGCAAATCATGCTGCGATACCTTA) and ferlavirusqAS2 (5'CTGATGGGAGATAATGCCTTGTCCTTCAT) (amplifies a 149 nucleotide segment of the polymerase (L) gene) (Hyndman et al., 2012) was used for FFPE material, while the primer set by Ahne (1999) (nested PCR that amplifies a 627 then 566 nucleotide segment of the L-gene) was used for swab and fresh-frozen tissue samples. For reovirus PCR, the hemi-nested primers 2334R/2090F and 2200R/2090F (amplifies a 292 then 162 nucleotide segment of the L1 genome segment) were used on sample types as previously described (Landolfi et al., 2010). An isolate of ferlavirus (American Type Culture Collection VR-1408) and *Nelson Bay orthoreovirus* (kindly donated by Professor Graham Wilcox, Murdoch University) served as positive controls.

Results

Clinical Signs

There were a variety of clinical signs in animals that were either PCR or virus isolation positive for Sunshine virus. Some infected animals displayed no overt signs of disease while for others, the clinical signs were neurological, neurorespiratory or non-specific (Tables 1 and 2). Neurological signs included head tremors, opisthotonus, incoordination, diminished righting reflexes, uncoordinated movement of the cranial and caudal body (Figure 1) and erratic mouth gaping. Respiratory signs included a mild discharge of clear viscous fluid from the mouth and dyspnoea. Non-specific signs included anorexia, stomatitis, weakness, lethargy, regurgitation and inappetence.

Polymerase Chain Reaction and Virus Isolation

Sunshine virus has been detected in fresh-frozen tissues (brain, kidney, lung and liver), formalin-fixed paraffin-embedded (FFPE) sections (various organs pooled), cloacal swabs, oral swabs and combined oral-cloacal swabs from three Australian python genera (Tables 1 and 2): *Antaresia*, *Aspidites* and *Morelia*.

From the fresh organ samples that were tested by PCR, Sunshine virus was detected most often in brain (11 out of 11 samples tested), followed by kidney (7/8), lung (8/11) and liver (5/8 for each). Similarly, for virus isolation, Sunshine virus was most often detected in samples of brain (4/10).

Of the 15 snakes that were positive for Sunshine virus by PCR *or* virus isolation (Table 1), fresh-frozen samples from 11 snakes (all seven from Collection 1 and all four from BVL Collection A) were used for both PCR *and* virus isolation. From the samples that were tested from these 11 snakes, a greater proportion of samples were positive by PCR (23 out of 27 or 85%, pooling the results for liver and kidney) than virus isolation (9 out of 27 or 33%).

Of the nine snakes that met the criteria to be included in the veterinary diagnostic laboratory cases, Sunshine virus could not be demonstrated by PCR in two snakes. These two cases were excluded from further study. In one of these cases, there were no histological abnormalities in the lungs although there were histological lesions in the brain consistent with those associated with Sunshine virus infection (detailed below). However, only paraffin-embedded brain tissue was available for PCR testing, and the head, including the brain, had been subjected to formalin containing 9% formic acid in order to decalcify the skull prior to histological processing. A second case that histologically had mild polioencephalomalacia involving the forebrain, was also negative for Sunshine virus by PCR on FFPE tissue. Neither ferlavirus nor reovirus could be detected by PCR in any of the snakes included in Table 1. Neither Sunshine virus nor ferlavirus could be detected by PCR in the six cases that were being used as negative controls for the histological examination of paramyxovirus-infected sections.

Combined oral-cloacal swab samples from snakes in Collection 2 were opportunistically taken over a 105-day period and tested for Sunshine virus by PCR. Of the 39 combined oral-cloacal samples that were collected from 23 snakes, five swabs, from five different snakes, were positive for Sunshine virus (Table 2). In three of these five snakes, individual (cloacal-only and oral-only) swabs were also collected and when the combined swab was positive, the individual swabs were also positive. Of particular note are spotted pythons 3-5 that tested positive for Sunshine virus, remained alive for the next sampling interval(s), and then tested negative (after which they were lost to follow-up). Spotted python 2 was symptomatic, tested negative on day 19, was later euthanased and organ samples were collected that tested positive (Table 1). The remaining 16 snakes in this collection that were tested, were asymptomatic and were only tested on days 45 and/or 105 (Supplementary Table 2). No sample from any of these snakes was positive for Sunshine virus.

Non-specific amplicons were produced occasionally when using the primer pair SunshineAS1-Sunshine S1 (153 nucleotide amplicon). On one occasion, a 167-nucleotide product was amplified and sequenced which was most closely related to an endonuclease from a lizard (*Anolis carolinensis*) (GenBank accession number: XM_003224137). Also with this primer pair, unsequenced amplicons that were approximately 400 and 900 nucleotides were often seen with organ sample total nucleic acid (DNA and RNA) templates that were negative for Sunshine virus. Non-specific amplicons have not been detected using the primer pairs SunshineAS2 (5’

CGGGATTCCCATAGAC)-SunshineS2 (230 nucleotides) or SunshineS2-SunshineAS1 (357 nucleotides). At least one appropriately-sized amplicon from each Sunshine virus PCR-positive snake was sequenced. In all cases (n=24), the amplicon was confirmed to be of Sunshine virus origin and single-base sequence variations were seen in two positions but both mutations were silent (see Supplementary Figure 2). No sequence variations were detected within a single collection.

Gross Pathology and Histology

Gross pathology and histology is reported only for snakes that were positive for Sunshine virus by either PCR or virus isolation.

All the Sunshine virus positive snakes from the veterinary diagnostic laboratory cases were in good to excellent body condition with moderately-sized to large coelomic fat bodies. Gross pathological findings were largely unremarkable and limited to mild or moderate pulmonary congestion and oedema in four snakes, and fibrinonecrotic exudate adherent to the oral mucosa in one snake.

The most consistent histological lesions of Sunshine virus positive snakes were in the brain. All cases exhibited mild to severe spongiosis of primarily the white matter of the hindbrain (Figures 2 and 3). In a minority of cases, the spongiosis also involved white matter tracts of the midbrain or the parenchyma of the cerebellum. In snakes with severe histological lesions, spongiosis and rarefaction of the parenchyma of the hindbrain extended the complete dorso-ventral height of the tissue and thus involved the intermingled grey

matter (Figure 3). In three of these severely affected snakes, neuronal chromatolysis or necrosis was evident in the hindbrain (Figure 3 inset). Mild to marked gliosis, composed of both astrocytosis and microgliosis, generally accompanied the spongiosis (Figures 2 and 3) and in four cases extended anteriorly to a lesser degree into the grey matter of the forebrain and olfactory bulb. Severely affected areas contained necrotic cell debris and low numbers of Gitter cells, primarily located in the meninges and surrounding parenchymal blood vessels. Lymphoplasmacytic perivascular cuffing and meningeal infiltration were prominent in only one snake. Axonal swellings and Wallerian degeneration were uncommon in the hindbrain. Intracytoplasmic eosinophilic or pale basophilic inclusion bodies were rarely observed in astrocytes, ependymal cells and the epithelium of the choroid plexus and in most cases deemed equivocal. The tinctorial properties of the inclusions tended to vary in the brain and in other tissues with the relative strength of the eosin or haematoxylin staining in the particular slide. In the two snakes in which cervical spinal cord was examined histologically, the tissue was normal in one snake while the other snake, which also had prominent hindbrain lesions, exhibited moderate spongiosis of the cervical white matter with accompanying Wallerian degeneration.

Five snakes exhibited changes indicative of mild to moderate bronchointerstitial pneumonia, variably including pulmonary septal and/or faveolar oedema or mild heterophil infiltration, mild to moderate hyperplasia, erosion or necrosis of the luminal respiratory

epithelium with associated patchy lymphoplasmacytic infiltration (Figure 4). One snake exhibited moderate diffuse type 2 pneumocyte hyperplasia. Rare equivocal intracytoplasmic inclusions were present in the respiratory epithelium, particularly in areas of eroded or necrotic epithelium. The most convincing possible viral inclusions were observed in the distal renal tubular system (primarily collecting ducts) in two snakes (Figure 5). In the five snakes in which spleen was examined, notable findings were mild to moderate lymphoid depletion in two snakes, marked lymphoid hyperplasia in one snake and a few small parenchymal heterophilic granulomas in another snake. All snakes exhibited a mild to moderate degree of macrovesicular vacuolation of hepatocyte cytoplasm (hepatic lipidosis, which is a common finding in captive snakes and considered incidental). Other miscellaneous histological findings were mild renal tubular degeneration or interstitial fibrosis in two snakes, mild to moderate colonic heterophil infiltration in two snakes and necrotising stomatitis in one snake. All other organs examined were histologically unremarkable.

Discussion

This report summarises the clinical signs, PCR results and pathological findings from the first clinical investigations of snakes infected with Sunshine virus.

Animals that were confirmed by PCR or virus isolation to be infected with Sunshine virus displayed a variety of clinical signs ranging from

no overt signs of disease to fulminant neurorespiratory disease with subsequent death. Non-specific clinical signs that are intermediate to these two extremes were also seen (e.g. weakness, lethargy and regurgitation). This suggests that the presence of neurorespiratory disease should alert the clinician to the possibility of Sunshine virus infection but also, the absence of clinical signs does not exclude the presence of this virus. These clinical findings are similar to a range of other primarily neurorespiratory diseases of snakes including ferlavirus, reovirus and inclusion body disease (reviewed by (Marschang and Chitty, 2004; Ritchie, 2006; Jacobson, 2007; Marschang, 2011)) and so these infections should also be considered in similarly-affected snakes.

Although this report is exploratory and is limited by a small number of samples that are PCR positive, our data support the recommendation that sampling the brain during a necropsy should form a priority sample during an investigation of suspected Sunshine virus infection. Similarly, our preliminary data suggest that (in order of preference) kidney, liver and lung samples may be useful when screening for this virus. There was little correlation between clinical signs (if any) and the organs that Sunshine virus was subsequently detected in by PCR so the data presented here suggests that clinical signs should not dictate sample selection. For example, we would still recommend testing the brain even in an animal without evidence of neurological clinical signs.

All of the serum samples that were PCR tested for Sunshine virus were negative, but considering the onset and duration of viraemia has not been determined, the significance of these results is undefined.

In our hands, PCR was able to positively detect Sunshine virus in pythons more often than virus isolation, therefore PCR is recommended for screening samples that may contain Sunshine virus. However, given that virus isolation does not rely on previously known sequence information, it continues to be an important tool of virus discovery. Two other papers have compared PCR to virus isolation in detecting reptilian paramyxoviruses. Both papers refer to the ferlaviruses. In a study by Kolesnikovas et al. (2006), using organ samples from three snakes infected with ferlavirus, both virus isolation (six positives out of seven samples) and PCR (three positives out of three samples) were able to detect infection in all three snakes. In a second study with a much larger data set, 203 samples (organs, swabs and tracheal washes) from 102 snakes were tested for the presence of ferlaviruses by PCR and virus isolation (Papp et al., 2010). From these 102 snakes, at least one organ sample was either PCR- or virus isolation-positive in 16 snakes (in our data set, fresh-frozen organ samples from 11 snakes, that were tested by virus isolation *and* PCR, were positive by PCR *or* virus isolation). Of these 16 snakes tested by Papp et al. (2010), 36 out of 51 organ samples were PCR-positive (70.6%) and three were positive by virus isolation (3/51 = 5.9%). Of the 11 snakes from our data set, from which multiple samples were tested by both PCR and virus

458 isolation, 23 out of 27 organ samples (pooling the results of liver and
459 kidney) were positive by PCR (85.2%) and nine were positive by virus
460 isolation (9/27 = 33.3%). There are several explanations for the
461 differential rates of detection of virus using PCR and virus isolation
462 between the Papp et al. (2010) study and ours, including: different
463 paramyxoviruses were of interest, the organs that were sampled
464 were not standardised and the PCR and virus isolation methods
465 were not the same. Despite these limitations, PCR detected the
466 reptilian paramyxovirus of interest far more reliably than virus
467 isolation in both investigations.

468 With respect to the oral/cloacal sampling of live animals in
469 Collection 2, spotted pythons 3 and 5 were PCR positive for
470 Sunshine virus but both were asymptomatic at the time of sampling
471 and were still alive and seemingly unaffected several months later.
472 For this reason, a positive PCR result for Sunshine virus should not
473 always be seen as a prelude to imminent death.

474 The diamond python and spotted python 2 that were exhibiting
475 neurological signs from Collection 2 both initially tested negative for
476 Sunshine virus by PCR yet later testing on both animals was positive.
477 This suggests that repeatedly testing animals for Sunshine virus may
478 be helpful in identifying infected snakes.

479 There is not yet enough data to provide an estimate for the
480 shedding duration of Sunshine virus. The interval between a positive
481 and a negative PCR result for spotted python 3 was 26 days and for
482 spotted pythons 4 and 5, the interval was 60 days. In all three cases,

483 the first PCR test result was positive so the start of viral shedding
484 cannot be determined. The shedding duration of another
485 paramyxovirus of snakes, ferlavirus, might extend for several
486 months (Lloyd and Flanagan, 1991) but for paramyxoviruses in
487 other species, the shedding periods are usually brief (Lamb and
488 Parks, 2007; Aldous et al., 2010; Dortmans et al., 2011; MacLachlan
489 and Dubovi, 2011). Experimentally transmitting Sunshine virus could
490 provide further insight into viral shedding dynamics.

491 Where a combined oral-cloacal swab was positive for Sunshine virus
492 by PCR and oral-only and cloacal-only swabs were available (n=3),
493 both the oral-only and cloacal-only swabs also tested positively. This
494 suggests that the mouth, the cloaca, or both sites combined, are all
495 appropriate areas to sample. This is limited by a small number of
496 cases and future work, in particular quantitative PCR, may provide
497 different conclusions.

498 In collection 2 there were also eight asymptomatic venomous
499 snakes that were not sampled, so the susceptibility of these genera
500 to Sunshine virus is unknown. Therefore, at this time, the range of
501 host species that are susceptible to Sunshine virus cannot be
502 reported beyond three genera of pythons: *Antaresia*, *Aspidites* and
503 *Morelia*.

504 Published descriptions of the brain pathology associated with
505 previously described paramyxoviruses in snakes are limited, with
506 most publications focusing on the lung pathology since respiratory
507 signs typically predominate in the infections (Jacobson et al., 1981;

Jacobson et al., 1997; Kolesnikovas et al., 2006; Jacobson, 2007). However, in the few publications where the brain histology is reported, lesions are variably described as absent, ballooning degeneration and demyelination of the brainstem, degeneration of axon fibers, gliosis, lymphohistiocytic meningoencephalitis, neuronal degeneration and few eosinophilic intracytoplasmic inclusions in glial cells (Jacobson et al., 1980; Jacobson et al., 1992; West et al., 2001). Thus the brain lesions seen in these cases associated with Sunshine virus infection include many of the histological features of other paramyxoviral infections in snakes. Further studies may show that the severe lesions predominantly affecting the hindbrain are unique features of Sunshine virus infection. From a comparative pathology standpoint it is notable how similar the brain lesions associated with Sunshine virus infection are to those due to canine distemper virus in which the brainstem is frequently the most severely affected region and white matter lesions of spongiosis (intramyelenic oedema) may predominate early in the infection with eventual development of nonsuppurative inflammation, demyelination and the presence of Gitter cells (Summers et al., 1995; Caswell and Williams, 2007; Zachary, 2007).

The respiratory lesions in snake paramyxovirus infections and canine distemper virus are described as bronchointerstitial pneumonia with hyperplasia of type 2 pneumocytes and variable neutrophilic/heterophilic and/or lymphocytic infiltrates, commonly with superimposed secondary bacterial bronchopneumonia (Jacobson et al., 1992; Jacobson et al., 1997; Kolesnikovas et al.,

2006; Caswell and Williams, 2007; MacLachlan and Dubovi, 2011). In the present cases of Sunshine virus, bronchointerstitial pneumonia was present in most cases although lesions were fairly mild, suggesting that Sunshine virus may be relatively less pneumotropic and more neurotropic compared to these other paramyxoviruses. Paramyxovirus infections may result in intracytoplasmic or intranuclear eosinophilic inclusions bodies in neurons, glial cells and a variety of epithelia, notably those of the respiratory and urinary systems (Caswell and Williams 2007, MacLachlan and Dubovy 2011, West et al 2001). In only a few cases of Sunshine virus infection were possible intracytoplasmic eosinophilic inclusions seen in the glial cells and respiratory epithelium, and those were considered equivocal since they occurred in areas of severe inflammation and could have represented fragments of necrotic cells or tissue, rather than viral inclusions. The most distinct intracytoplasmic inclusions seen in these Sunshine virus cases were in the otherwise normal renal distal tubule and collecting duct epithelia. Future work should include transmission electron microscopy of the inclusions seen in Sunshine virus, to determine if they are of viral origin.

Along with paramyxoviral infection, inclusion body disease (IBD) is another infectious disease of snakes that is capable of causing neurological signs (Schumacher et al., 1994; Jacobson et al., 2001; Vancraeynest et al., 2006; Jacobson, 2007; Chang and Jacobson, 2010). Neurohistopathology associated with this syndrome includes diffuse spongiosis, demyelination, neuronal degeneration, gliosis

and nonsuppurative or lymphoplasmacytic meningoencephalitis (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Jacobson et al., 2001; Vancraeynest et al., 2006). The histopathology associated with Sunshine virus infection overlaps somewhat with that associated with IBD. However, in most cases of IBD, bright eosinophilic intracytoplasmic inclusions are readily appreciable within neurons and in abundance in epithelial cells in a variety of tissues (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Chang and Jacobson, 2010), a feature that was not present in the Sunshine virus cases detailed here.

Reoviruses have been either identified by electron microscopy or isolated from various snakes, some with clinical signs of neurological dysfunction (Ahne et al., 1987; Vieler et al., 1994; Rose et al., 2005; Abbas et al., 2011). Unfortunately, the brain was not examined histologically in these reports, therefore any neuropathology that is associated with reovirus infection remains undescribed. Finally, an intranuclear inclusion disorder has been described in *Morelia* sp. that was associated with writhing and bloating (Boyer et al 2000). Electron microscopy of the brain revealed accumulations of particles that resembled retroviruses. However, the defining histological lesion in this syndrome was abundant prominent eosinophilic or amphophilic intranuclear inclusion bodies in glial cells in the brain (described in (Boyer et al., 2000) and images reproduced in (Ritchie, 2006)), inconsistent with the rare, equivocal intracytoplasmic inclusions in glial cells in these Sunshine virus cases.

585 We recommend three future areas of research that may enhance
586 the understanding of the disease that is associated with Sunshine
587 virus.

588 Firstly, investigations should be pursued that might help determine
589 if Sunshine virus has a causative relationship with disease. Although
590 the histopathological findings reported here are consistent with
591 paramyxoviral pathology and are explanatory for the clinical signs
592 seen, a causative link between infection and disease has not yet
593 been investigated through immunohistochemistry, *in situ*
594 hybridisation or a transmission study.

595 Secondly, the non-degenerate primer sets described in this report
596 should be reviewed. This is particularly important considering the
597 minimal sequence variation that has been identified in the
598 sequenced amplicons to date. Although the minimal sequence
599 variation may be explained by the highly conserved part of the
600 paramyxoviral genome (the polymerase gene, (Kurath et al., 2004))
601 that was amplified, the ability of these primers to detect different
602 strains of Sunshine virus has not been demonstrated and so the
603 application of these primers for diagnostic use may be limited.
604 Degenerate and/or (hemi)nested primer sets may improve the
605 detection capabilities for Sunshine virus possibly revealing closely
606 related viruses.

607 Lastly, the screening of animals may be improved by testing that is
608 capable of detecting an immune response to infection. Humoral
609 assays (e.g. haemagglutination inhibition and virus neutralisation)

are already available at a selection of diagnostic laboratories for other reptilian viruses (Heard et al., 2004) and antibody testing could assist the veterinarian's decision making process concerning the healthcare of these animals.

Conclusion

Sunshine virus is a recently discovered novel paramyxovirus of Australian pythons. This paper presents data based on opportunistic testing on a limited number of naturally-infected snakes from multiple collections. Within this framework, the following preliminary conclusions can be made regarding snakes infected with Sunshine virus: some snakes do not display any clinical signs of disease while for others, the signs are non-specific and/or localised to the neurorespiratory systems. Gross pathology is often unremarkable. The most reliable histopathological finding is spongiosis of primarily the white matter of the hindbrain. Given that there is still much to be discovered regarding the pathogenesis and infection dynamics of Sunshine virus, at necropsy, the investigation should involve histopathological examination in conjunction with PCR testing. PCR testing seems to be more rewarding on tissues than oral/cloacal swabs. Our results suggest that (in order of preference) the brain, kidney, lung and liver are all priority necropsy samples but there is currently no data on other tissue samples and therefore all tissues should be sampled until supported recommendations suggest otherwise. In live animals, there appears to be benefit in testing swabs by PCR on more than one occasion.

Detecting Sunshine virus by PCR on a swab sample should not be seen as a prelude to imminent death but the value of an individual that is possibly shedding virus should be weighed up against the value of a collection.

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Tables

Table 1. PCR and virus isolation results from snakes that were positive for Sunshine virus by either test. Positive virus isolation was defined by characteristic cytopathic effect (large syncytium with minimal cell lysis). - = negative. + = positive. n.s. = not sampled. n.t. = not tested. BVL = Berrimah Veterinary Laboratory. * = PCR performed on pooled formalin-fixed paraffin-embedded (FFPE) organs that included at least brain and lung. Unless otherwise stated, all organ samples were fresh or freshly-frozen. All snakes were euthanased on either humane grounds or as part of a disease investigation being undertaken by the attending veterinarian. Information on exposure and mating among snakes relates to additional historical information on the snake collection provided in Supplementary Table 1.

Species, Sex	**Sample origin**	**Clinical History**	**Polymerase Chain Reaction (PCR)**						**Virus Isolation**		
			Brain	Lung	Liver	Kidney	Serum	Various FFPE organs*	Brain	Lung	Kidney-Liver
Jungle carpet python 1, male (*Morelia spilota cheynei*)	Collection 1	Clinical respiratory disease. Mated to jungle carpet pythons 7 and 10.	+	-	-	+	-	+	-	-	-
Jungle carpet python 2, female	Collection 1	Slight head tremor. Mated to jungle carpet python 6	+	+	+	+	n.s.	n.t.	-	-	-
Jungle carpet python 3, female	Collection 1	Slight head tremor. Mated to jungle carpet python 6	+	+	+	+	-	n.t.	-	-	-
Jungle carpet python 4, female	Collection 1	Subtle neurological signs. Shared enclosure with snake that died acutely. Mated to jungle carpet python 5	+	-	+	+	-	n.t.	+	-	+
Black-headed python 1, female (*Aspidites melanocephalus*)	Collection 1	Low grade respiratory disease and stomatitis. Shared enclosure with two snakes (one after the other) that died acutely	+	+	+	+	-	n.t.	+	+	+

Black-headed python 2, male	Collection 1	Asymptomatic. Mated to snake that later died acutely	+	-	-	-	-	n.t.	-	-	-
Woma python 1, female (*Aspidites ramsayi*)	Collection 1	Chronic respiratory signs and periods of neurological dullness. Mated to woma python 3	+	+	+	+	-	n.t.	-	-	-
Spotted python 2, sex unknown (*Antaresia maculosa*)	Collection 2	Weakness and lack of coordination	+	+	-	+	n.s.	n.s.	n.t.	n.t.	n.t.
Jungle carpet python 1, male	BVL Collection A	Several snakes from collection A showed dysecdysis, anorexia, neurological signs, death over several weeks. This snake was euthanased while still relatively active	+	+	n.s.	n.s.	n.s.	+	+	+	n.s.
Jungle carpet python 2, male	BVL Collection A	Died naturally	+	+	n.s.	n.s.	n.s.	-	-	+	n.s.
Jungle carpet python 3, female	BVL Collection A	Moribund	+	n.s.	n.s.	n.s.	n.s.	+	+	n.s.	n.s.
Carpet python, male (*Morelia spilota*)	BVL Collection A	Neurological signs and dyspnoea	n.s.	+	n.s.	n.s.	n.s.	+	n.s.	-	n.s.
Carpet python 1, male	BVL Collection B	Neurological signs	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.
Carpet python 2, male	BVL Collection B	Delayed righting reflex, incoordination, gaping mouth	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.
Carpet python 3, male	BVL Collection B	Opisthotonus, loss of righting reflex, anorexia of 2-3 months duration	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.
Total positive / total samples tested			**11/11**	**8/11**	**5/8**	**7/8**	**0/6**	**7/8**	**4/10**	**3/10**	**2/7**

804

Table 2. PCR results of combined oral-cloacal swabs from Collection 2 taken over a 105-day period. Only snakes that died or were positive for Sunshine virus by PCR are presented. - = negative. + = positive. n.s. = not sampled. * = dead prior to this sampling date. a = organ samples not retrieved for further testing. b = organ samples retrieved for further testing and results presented in Table 1. # = separate cloacal-only and oral-only swabs were also PCR positive. n/a = not applicable.

Species	Clinical History	day 0	day 19	day 45	day 105
Spotted python 1 (*Antaresia maculosa*)	Asymptomatic	-	n.s.	deceased*a	n/a
Diamond python (*Morelia spilota spilota*)	Weakness, regurgitation, decreased righting reflex	-	+	deceased*a	n/a
Spotted python 2	Weakness, lack of coordination	n.s.	-	euthanased*b	n/a
Spotted python 3	Asymptomatic	n.s.	+	-	-
Spotted python 4	Weakness	n.s.	n.s.	+#	-
Spotted python 5	Asymptomatic	n.s.	n.s.	+#	-
Coastal carpet python 1 (*Morelia spilota mcdowelli*)	Weakness, lethargy, inappetence	n.s.	n.s.	+#	n.s.

Figure Captions

Figure 1. Neurological signs in two Australian snakes. Both snakes were later confirmed to be infected with Sunshine virus by PCR (unpublished). **Above:** Diamond python (*Morelia spilota spilota*) with diminished righting reflex and abnormal rigidity of caudal half of body. **Below:** Centralian carpet python (*Morelia bredli*) with abnormal posture. Photos courtesy of Robyn Kollbaum.

Figure 2. Dorsal hindbrain, Berrimah Veterinary Laboratories, Collection A, jungle carpet python 1. Moderate patchy spongiosis involving primarily the white matter of the hindbrain (HB) and to a mild degree, the granular layer of the cerebellum (CB). Note mild degree of associated gliosis and lack of inflammatory cellular infiltrate in the meninges (M). CP= choroid plexus in the fourth ventricle. Haematoxylin and eosin stain. Bar = 200 µm.

Figure 3. Brainstem, Berrimah Veterinary Laboratories, Collection B, carpet python 2. Severe spongiosis involving the complete dorsoventral height of the hindbrain (HB) with relative sparing of the cerebellum (CB), midbrain (MB, including optic tectum (OT)), cranial extent of the spinal cord (SP) and caudal extent of the forebrain (FB). The rectangle outlines the area of the inset in which moderate gliosis and neuronal necrosis (arrowheads) are evident. Haematoxylin and eosin stain. Main bar = 500 µm. Inset bar = 50 µm.

834 **Figure 4.** Lung, Berrimah Veterinary Laboratories, Collection A,
835 carpet python. Mild hyperplasia and jumbling of the luminal
836 respiratory epithelium (LRE) with scattered necrotic cells and
837 associated moderate lymphoplasmacytic infiltration. Generalised
838 moderate vascular congestion and mild septal (S) oedema. The few
839 red blood cells in the air spaces (faveolae) between septae are
840 interpreted as post-mortem artefact rather than pre-mortem
841 haemorrhage. Haematoxylin and eosin stain. Bar = 50 µm.

842 **Figure 5.** Renal collecting duct, Berrimah Veterinary Laboratories,
843 Collection A, jungle carpet python 3. Basophilic intracytoplasmic
844 inclusion bodies in epithelial cells (arrows). Haematoxylin and eosin
845 stain. Bar = 10 µm.

Figure 1 above

Figure 1 below

Figure 2 colour

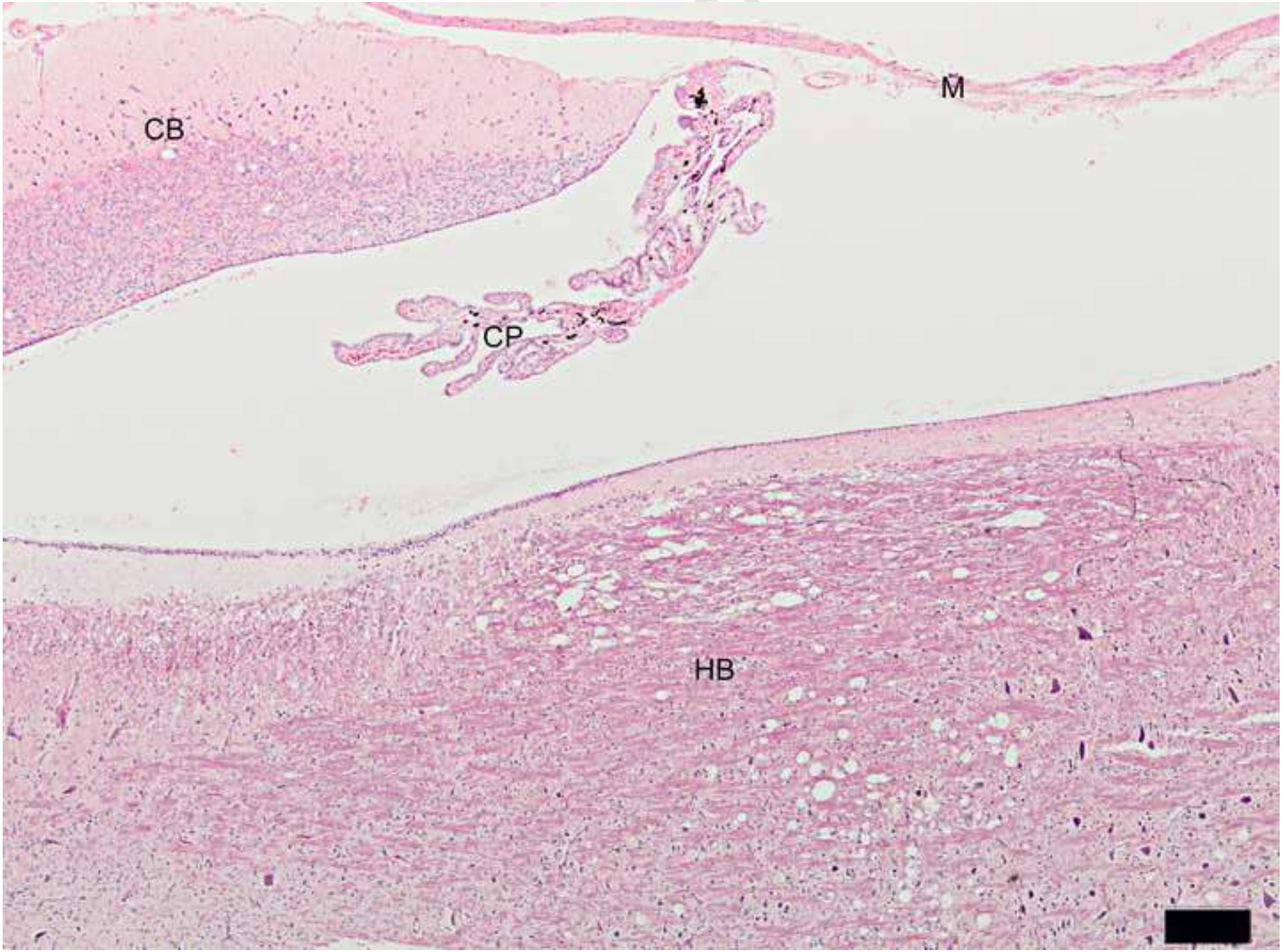

Figure 3 colour

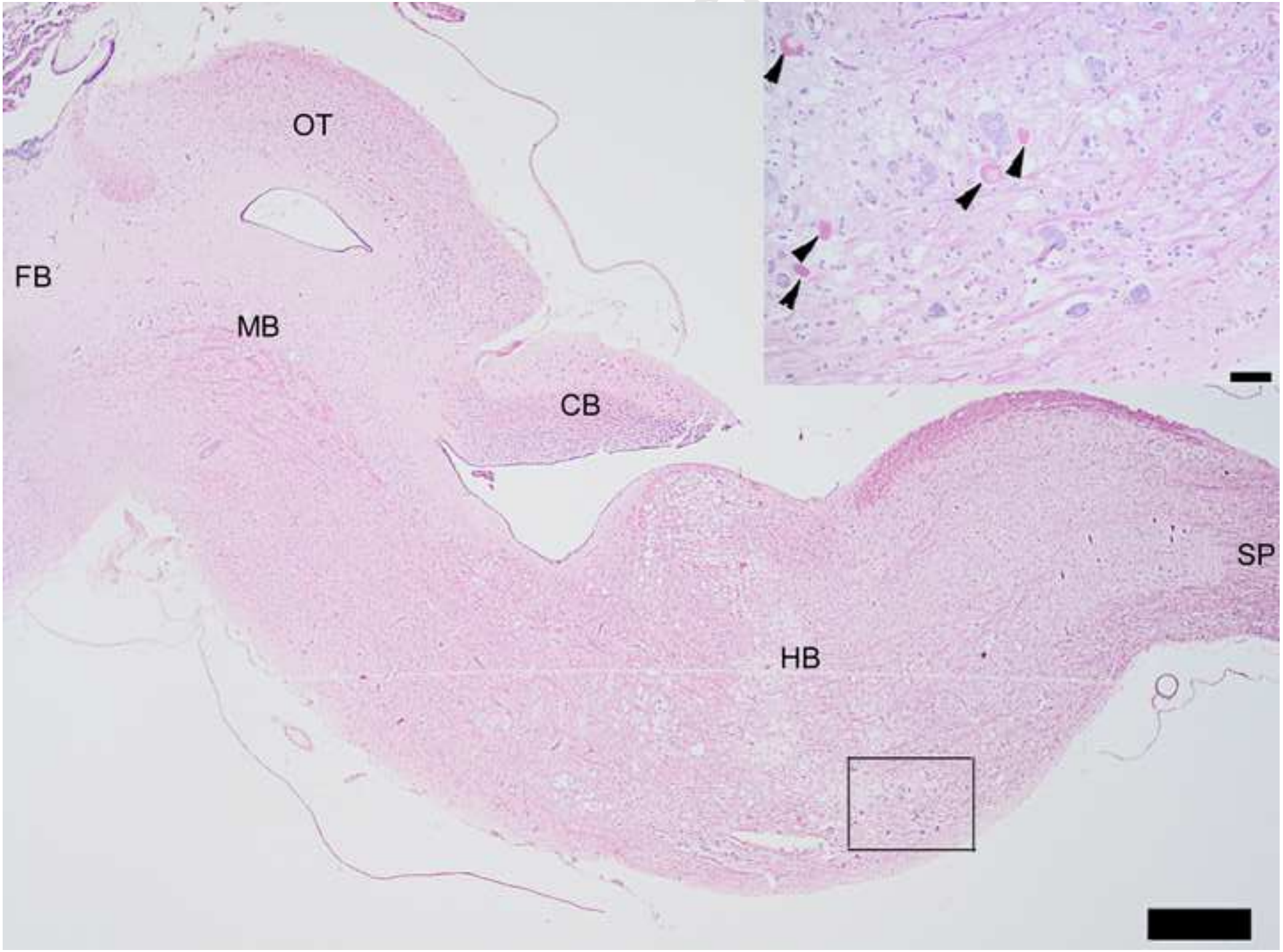

Figure 4 colour

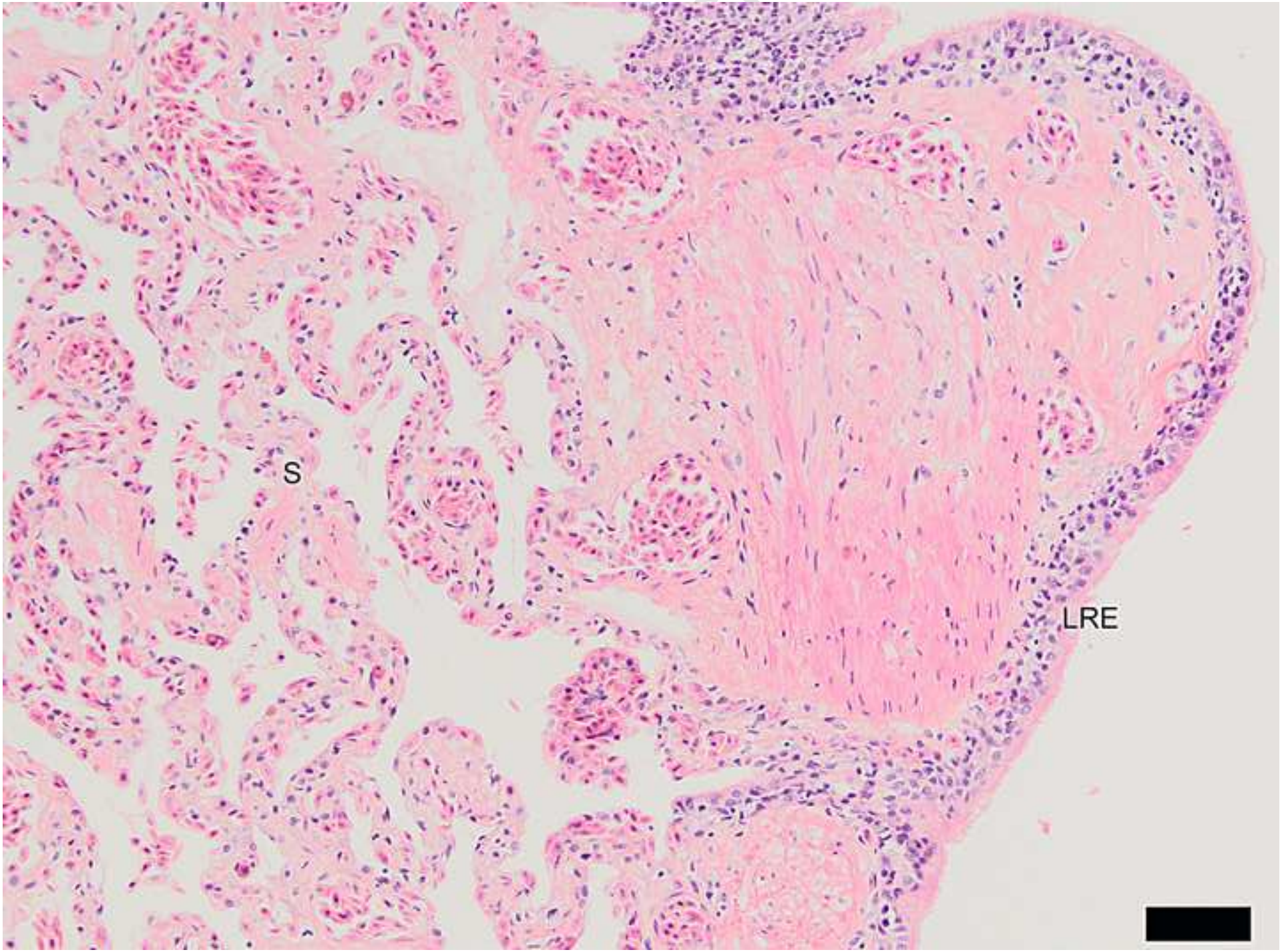

Figure 5 colour